AUTOTAXIN: MOTILITY STIMULATING PROTEIN USEFUL IN CANCER DIAGNOSIS AND THERAPY

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Field of the Invention

The present invention relates, in general, to a motility stimulating and compositions comprising the same. In particular, the present invention relates to a purified form of the protein and peptides thereof, for example, autotaxin (herein alternative referred to as "ATX"); a DNA segment encoding autotaxin; recombinant DNA molecules containing the DNA segment; cells containing the recombinant DNA molecule; a method of producing autotaxin; antibodies to autotaxin; and methods of cancer diagnosis and therapy using the above referenced protein or peptides thereof and DNA segments.

Background of the Invention

Cell motility plays an important role in embryonic events, adult tissue remodeling, wound healing, angiogenesis, immune defense, and metastasis of tumor cells (Singer, 1986). In normal physiologic processes, motility is tightly regulated. On the other hand, tumor cell motility may be aberrantly regulated or autoregulated. Tumor cells can respond in a motile fashion to a variety of agents. These include host-derived factors such as scatter factor (Rosen, et al., 1989) and growth factors (Kahan, et al., 1987; Stracke, et al.; Tamm, et al., 1989; Wang, et al. 1990; and Jouanneau, et al. 1991), components of the extracellular matrix (McCarthy, et al. 1984), and tumor-secreted or autocrine factors (Liotta, et al. 1988; Ruff, et al. 1985; Atnip, et al. 1987; Ohnishi, et al. 1990; Silletti, et al. 1991; and Watanabe, et al. 1991).

Many types of host-derived soluble factors act in a paracrine fashion to stimulate cell locomotion.

Motility-stimulating proteins called "scatter factors" have been identified which are produced by embryonic fibroblasts and by smooth muscle cells (Stoker, et al. 1987). Scatter factors stimulate random and directed motility by epithelial cells, keratinocytes, vascular endothelial cells and carcinoma cells (Stoker, et al. 1987; Rosen, et al. 1990; and Weidner, et al. 1990), but not fibroblasts. In addition, a number of host-secreted growth factors have been demonstrated to stimulate motility in tumor cells, including nerve growth factor (Kahan, et al. 1987) insulin-like growth factor-I (Stracke, et al. 1988), interleukin-6 (Tamm, et al. 1989), interleukin-8 (Wang, et al. 1990), and acidic fibroblast growth factor (Jouanneau, et al. 1991). These paracrine factors may influence "homing" or the directionality of tumor cell motility.

In contrast to these host-derived factors, many types of tumor cells have been found to produce proteins termed "autocrine motility factors" which stimulate motility by the same tumor cells which make the factor (Liotta, et al. 1986). Autocrine motility factors are not specific for a given type of cancer cell but have a wide spectrum of activity on many types of cancer cells (Kohn, et al. 1990), with little effect on normal fibroblasts or leukocytes.

Autocrine motility factors identified to date act through cell-surface receptors (Stracke, et al. 1987; Nabi, et al. 1990; Watanabe, et al. 1991) resulting in pseudopodial protrusion (Guirguis, et al. 1987) leading to both random and directed migration (Liotta, et al. 1986; Atnip, et al. 1987; Ohnishi, et al. 1990).

Prior studies of human A2058 melanoma cells have demonstrated that these cells are a particularly rich source of autocrine motility factors. An autocrine motility factor with a molecular mass of approximately 60 kDa has been previously isolated from the conditioned

media of these cells. (Liotta, et al. 1986). Similar tumor cells derived or induced factors with the same molecular weight have subsequently been reported and purified by several investigators (Atnip, et al. 1987; Schnor, et al. 1988; Ohnishi, et al. 1990; Silletti, et al. 1991; Watanabe et al. 1990). Such factors are thought to play a key role in tumor cell invasion.

Most of the motility factors identified to date have not been purified to homogeneity and have not been sequenced. The novel tumor motility factor of the present invention, named herein as autotaxin ("ATX"), has been purified and verified to be a homogeneous sample by two-dimensional gel electrophoresis. The protein of the present invention is unique from any previously identified or purified motility factor. The molecular size of ATX is about 125 kilo Daltons ("kDa") and it has an isoelectric point of approximately 7.7. ATX stimulates both random and directed migration of human A2058 melanoma cells at picomolar concentrations. The activity of the ATX factor is completely sensitive to inhibition by pertussis toxin. No significant homology has been found to exist between the protein of the invention and any mammalian protein including previous factors known to stimulate cell motility.

There is a great clinical need to predict the aggressiveness of a patient's individual tumor, to predict the local recurrence of treated tumors and to identify patients at high risk for development of invasive tumors. The present invention provides a functional marker which is functionally related to the invasive potential of human cancer. The invention further provides an assay for this secreted marker in body fluids, or in tissues. The assay of the invention can be used in the detection, diagnosis, and treatment of human malignancies and other inflammatory, fibrotic, infectious or healing disorders.

SUMMARY OF THE INVENTION

The present invention relates, generally, to a motility stimulating protein and corresponding peptides thereof, and to a DNA segment encoding same. A human cDNA clone encoding a tumor cell motility-stimulating protein, herein referred to as autotaxin or "ATX", reveals that this protein is an ecto/exoenzyme with significant homology to the plasma cell membrane differentiation antigen PC-1. ATX is a 125 kDa glycoprotein, previously isolated from a human melanoma cell line (A2058), which elicits chemotactic and chemokinetic responses at picomolar to nanomolar concentrations.

It is a specific object of the present invention to provide autotaxin and peptide fragments thereof.

It is a further object of the present invention to provide a DNA segment that encodes autotaxin and a recombinant DNA molecule comprising same. It is a further object of the present invention to provide a cell that contains such a recombinant molecule and a method of producing autotaxin using that cell.

Another object of the present invention is the identification of a transmembrane domain of the human liver autotaxin protein and its apparent absence in tumorous forms of autotaxin. The tumorous form of autotaxin appears to be a secreted protein. The present invention relates to utilization of the different sites of localization for diagnosis and prognosis of the stages of tumor progression. Further, the invention relates to treatment methods, designed to advantageously block the secreted form of autotaxin activity while having little effect on the membrane-bound form of autotaxin.

Yet another object of the present invention relates to the identification of a highly variable region within the autotaxin gene, called a "hot spot". The variations in sequence apparently result in mutations, insertions, deletions and premature termination of

translation. The present invention relates to manipulating this region so as to alter the activity of the protein. Further, the hot spot can serve as a marker in tumor diagnosis differentiating between different forms of the autotaxin protein.

It is yet another object of the present invention to provide a method of purifying autotaxin.

It is a further object of the present invention to provide cloned DNA segments encoding autotaxin and fragments thereof. The cDNA encoding the entire autotaxin protein contains 3251 base pairs, and has an mRNA size of approximately 3.3 kb. The full-length deduced amino acid sequence of autotaxin comprises a protein of 915 amino acids. Database analysis of the ATX sequence revealed a 45% amino acid identity (including 30 out of 33 cysteines) with PC-1, a pyrophosphatase/type I phosphodiesterase expressed on the surface of activated B cells and plasma cells. ATX, like PC-1, was found to hydrolyze the type I phosphodiesterase substrate p-nitrophenyl thymidine-5'monophosphate. Autotaxin now defines a novel motility-regulating function for this class of ecto/exo-enzymes.

Further objects and advantages of the present invention will be clear from the description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Fractionation of ATX by hydrophobic interaction. A 200 ml sample of A2058 conditioned media was chromatographed on a 200 mL column of phenyl Sepharose-4B. Buffer A was 50 mM Tris (pH 7.5), 5% methanol, and 1.2 M ammonium sulfate. Buffer B was 50 mM Tris (pH 7.5), 5% methanol and 50% ethylene glycol. The gradient (----) represents a double linear gradient with decreasing ammonium sulfate (1.2 to 0.0 M) and increasing ethylene glycol (0 to 50%). Absorbance was monitored at 280 nm (———) and indicated that most of the protein did not bind to the column. Ten ml fractions were assayed for

motility stimulating capacity using the Boyden Chamber assay (o). The peak of motility activity occurred between 900 and 1050 minutes, ~ 12% of the gradient.

Figure 2. Isolation of ATX by lectin affinity chromatography. 20 ml portions of the phenyl Sepharose activity peak were affinity purified on a 40 ml Concanavalin A Affi-Gel column. The bound components were eluted with a step gradient (----) of methyl α-D-mannopyranoside (0.0 mM, 10 mM, and 500 mM) in a buffer consisting of 0.05 M Tris (pH 7.5), 0.1 M NaCl, 0.01 M $CaCl_2$ and 20% ethylene glycol. Absorbance was monitored at 280 nm (_____) and indicated that the majority of the protein components did not bind to the column. Motility was assayed in 10 mL fractions (...o...) and was found predominantly in the 500 mM elution concentration. One of seven chromatographic runs is shown.

Figure 3. Purification of ATX by weak anionic exchange chromatography. Approximately 30% of the activity peak eluted from the Con A affinity column was applied to a ZORBAX BioSeries-WAX column. The bound components were eluted with an NaCl gradient (----) in a buffer consisting of 10 mM Tris (pH 7.5) and 30% ethylene glycol. Motility (o) was assayed in 1.0 ml fractions. The peak of activity eluted in a discrete but broad region in the shallow portion of the gradient. Absorbance was monitored at 230 nm (_____). The majority of the protein components not associated with activity bound strongly to the column were eluted at 1.0 M NaCl. One of two chromatographic runs is shown.

Figure 4. Purification of ATX by molecular sieve exclusion chromatography. The entire activity peak eluted from the weak anion exchange column was applied to a series of TSK columns (4000SW, 4000SW, 3000SW, and 2000SW, in this order). Proteins were eluted in a buffer consisting of 0.1M $NaPO_4$ (pH 7.2) with 10% methanol and 10% ethylene glycol. Two major protein peaks were evident

by monitoring the absorbance at 235 nm (_____). Motility (...o...) was assayed in 0.4 ml samples and found predominantly in the first, smaller, protein peak.

Figure 5. Final purification of ATX by strong anionic exchange chromatography. Approximately 15% of the activity peak from the molecular sieve exclusion series was applied to a Pro-Pac PA1 column. Protein which bound to the column was eluted with a NaCl gradient (----) in a buffer consisting of 10 mM Tris (pH 7.5), 5% methanol and 20% ethylene glycol. Absorbance was monitored at 215 nM (_____). Motility activity was assayed in 1.0 ml fractions at two different dilutions: 1/5 (...o....) or 1/15 (.__.o.__.). Activity was found to correspond to a double protein peak in the central region of the gradient.

Figure 6A, 6B and 6C. Protein components associated with the activity peaks from various stages of purification. The activity peak from each chromatographic fractionation was pooled, concentrated and analyzed by SDS-polyacrylamide gel electrophoresis. Molecular weight standards are in Lane 1 for each panel. Panel 6A) 8-16% gradient gel of the first three purification steps, run under non-reducing conditions. Lane 2 is an aliquot of the pooled activity peak eluted from the phenyl sepharose fractionation. Lane 3 is an aliquot of the pooled activity peak eluted from the Con A affinity purification. Lanes 4 and 5 show the "peak" and "shoulder" of activity fractionated by weak anion exchange chromatography (Figure 3). Panel 6B) 7% gel of the activity peak fractionated by molecular sieve exclusion chromatography. Lanes 2 and 3 show the protein separation pattern of the total pooled activity peak when the gel was run under non-reducing and reducing conditions, respectively. Panel 6C) 8-16% gradient gel of the final strong anionic exchange chromatographic separation, run under non-reducing conditions. Lane 2 comprises ~1% of the total pooled activity peak eluted from the column.

Figure 7. Two-dimensional gel electrophoresis of ATX. Purified ATX (Figure 6, Panel C) was subjected to non-equilibrium isoelectric focusing (5 hr. at 500v), then applied to a 7.5% SDS-polyacrylamide gel for the second dimension. The pH separation which resulted was measured in 0.5 cm samples of concurrently run tube gels and is shown at the top. Molecular weight standards for the second dimension are shown on the right. This analysis reveals a single component with pI = 7.7 ± 0.2 and M_r = 120,000.

Figure 8. Dilution curve of ATX. Purified ATX (Figure 6, Panel C) was serially diluted and tested for motility-stimulating activity. The result, with unstimulated background motility subtracted out, shows that activity is half-maximal at ~ 500 pM ATX.

Figure 9. Pertussis toxin (PT) sensitivity of ATX. A2058 cells were pre-treated for 1 hr. prior to the start of the motility assay with 0.5 μg/ml PT in 0.1% BSA-DMEM or with 0.1% BSA-DMEM alone (for untreated control). The motility activity stimulated by purified ATX (Figure 6, Panel C) was then assessed for the two treatment groups. The result, expressed as cells/HPF ± S.E.M. with unstimulated background motility subtracted out, reveals profound inhibition of PT-treated cells (hatched) compared to untreated cells (solid). PT had no effect on cell viability. S.E.M.'s were < 10%.

Figure 10. Checkerboard analysis of ATX-stimulated motility. Varying dilutions of autotaxin were added to the upper chamber with the cells and/or to the lower chamber, as shown. Motility response, expressed as cells/HPF ± S.E.M., was assessed for each point in the checkerboard.

Figure 11. Purification of ATX peptides on HPLC. ATX, purified to homogeneity by strong anionic exchange chromatography, was sequentially digested by cyanogen bromide, subjected to reduction and

pyridylethylation, and digested by trypsin. The resulting peptides were purified on an Aquapore RP300 C-8 reverse phase column using a (0-70)% acetonitrile gradient in 0.1% trifluoroacetic acid (----). The absorbance was monitored at 215 nm (_____) and peaks were collected. Seven peaks, chosen at random for N-terminal amino acid sequence analysis, are shown with appropriate numbers.

Figure 12. Cloning Strategy, schematically depicted.

Figure 13. Schematic Diagram of autotaxin gene region.

For A2058: 4C11 is the original DNA clone obtained by screening an A2058 cDNA expression library in λgt11 with anti-peptide ATX-102. Upstream ATX peptide sequences were utilized for PCR amplification of A2058 mRNA, using the technique of reverse transcription/PRC. These peptides include ATX-101, ATX-103, and ATX-224. The approximate localization of each of peptide was obtained by matching the peptide with its homologous region on PC-1.

For N-tera 2D1, a λgt1 0 cDNA library was amplified and the cDNA inserts were isolated. PCR amplification, based on homologies with A2058 sequence, was utilized for DNA sequencing.

For normal human liver, a mRNA from liver was amplified with 5'RACE using primers from the known ATX-224 region of A2058 and N-tera 2D1.

Figure 14. Schematic match-up of ATX peptides with putative A2058 protein sequence.

Figure 15. Schematic match-up of ATX peptides with putative N-tera 2D1 protein sequence.

Figure 16: ATX Treatment with PGNase F. Partially purified ATX was treated with 60 mU/ml PNGase F at 37°C for 16 hr under increasingly denaturing conditions. The treated ATX samples were separated by SDS polyacrylamide gel electrophoresis run under reducing

conditions and stained with Coomassie blue G-250. Lane 1 contains untreated ATX (arrow) with no enzyme added. Lane 2 contains the reaction mixture run under non-denaturing conditions (50 mM tris/10% ethylene glycol, pH 7).. Lanes 3 and 4 have added 0.1 M β-mercaptoethanol and 0.5% Nonidet-P40, respectively. Lanes 5 and 6 contain the reaction mixtures in which the ATX sample was first boiled for 3 min in 0.1% SDS with (lane 6) or without (lane 5) 0.1 M β-mercaptoethanol, then had 0.5% Nonidet-P40 added to prevent enzyme denaturation. The enzyme can be detected as an ~44 kDa band in lanes 2-6.

Figure 17: Effect of varying concentrations of PNGase F on ATX molecular weight and motility-stimulating activity. Partially purified ATX was treated with various concentrations (range 0 - 60 mU/ml, shown on horizontal axis) of PNGase F at 37°C for 16 hr. Figure 17A shows the effect of the different treatments on ATX molecular weight. At concentrations of enzyme $\geq$ 30 mU/ml, the deglycosylation reaction appears to be complete. Figure 17B shows the effect of the identical reaction mixtures on motility-stimulating capacity (immediately below the corresponding protein band of Figure 17A). There is no significant difference between any of the treatment groups.

Figure 18: Comparison of amino acid sequences of ATX (SEQ ID NO:69) and PC-1. The amino acid sequences of ATX and PC-1 (Bucklay, et al., 1990) are compared. Amino acid identity is indicated by a vertical line between the sequences. The location of the putative transmembrane/signal sequence is shown by a solid line. The two somatomedin B domains are identified by dashed lines. The putative phosphodiesterase active site is indicated by emboldened lines. The loop region of a single EF hand loop region is identified with double lines. The presumed cleavage site for each protein is indicated with arrows.

Figure 19: Domain structure of ATX and PC-1.

Putative domains are indicated for the two homologous proteins, ATX and PC-1.

DETAILED DESCRIPTION OF THE INVENTION

Tumor cell motility is a critical component of invasion and metatasis, but the regulation of this motility is still poorly understood. At least some tumor cells secrete autocrine motility factors (AMF's) that stimulate motility in the producing cells. Like the analogous autocrine growth factors, these AMF's allow tumor cells independence from the host in this important component of the metastatic cascade. One AMF, autotaxin (ATX), has recently been purified to homogeneity from the human melanoma cell line, A2058 (Stracke, et al., 1992). The purified protein was enzymatically digested and the peptide fragments were separated by reverse phase HPLC. A number of these peptides have been sequenced by standard Edman degradation (Table 6) from different purifications and different enzymatic digestion. Sequence information, obtained initially on 19 purified tryptic peptides, confirmed that the protein is unique with no significant homology to growth factors or previously described motility factors. These peptide sequences have now been used as the basis for identifying and sequencing the cDNA clone for ATX. The present invention comprises an amino acid sequence of ATX as well as a nucleic acid sequence coding for the ATX protein.

TABLE 6. PEPTIDE SEQUENCES FOR AUTOTAXIN.

PEPTIDE NO.	AMINO ACID SEQUENCE	SEQ ID: NO:
ATX-18	WHVAR	SEQ ID NO:1
ATX-19	PLDVYK	SEQ ID NO:2
ATX-20	YPAFK	SEQ ID NO:3
ATX-29	PEEVTRPNYL	SEQ ID NO:5

T,120

ATX-34B	RVWNYFQR	SEQ ID NO:38
ATX-41	HLLYGRPAVLY	SEQ ID NO:29
ATX-48	VPPFENIELY	SEQ ID NO:7
ATX-59	TFPNLYTFATGLY	SEQ ID NO:32
ATX-100	GGQPLWITATK	SEQ ID NO:8
ATX-101/223A	VNSMQTVFVGYGPTFK	SEQ ID NO:9
ATX-102	DIEHLTSLDFFR	SEQ ID NO:10
ATX-103	TEFLSNYLTNVDDITLVPETLGR	SEQ ID NO:11
ATX-104	VNVISGPIDDYDYDGLHDTEDK	SEQ ID NO:33
ATX-204	MHTARVRD	SEQ ID NO:39
ATX-205	FSNNAKYD	SEQ ID NO:40
ATX-209	VMPNIEK	SEQ ID NO:41
ATX-210	TARGWECT	SEQ ID NO:42
ATX-212	(N)DSPWT(N)ISGS	SEQ ID NO:43
ATX-214	LRSCGTHSPYM	SEQ ID NO:44
ATX-215/34A	TYLHTYES	SEQ ID NO:45
ATX-213/217A	AIIANLTCKKPDQ	SEQ ID NO:46
ATX-216	IVGQLMDG	SEQ ID NO:47
ATX-218/44	TSRSYPEIL	SEQ ID NO:48
ATX-223B/24	QAEVSSVPD	SEQ ID NO:49
ATX-224	RCFELQEAGPPDDC	SEQ ID NO:50
ATX-229	SYTSCCHDFDEL	SEQ ID NO:51
ATX-244/53	QMSYGFLFPPYLSSSP	SEQ ID NO:52

ATX is a glycosylated protein due to its high affinity for concanavalin A and amino acid sequence analysis of the ATX peptides. ATX has been demonstrated to be a 125kDa glycoprotein whose molecular weight reduced to 100-105kDa after deglycosylation with N-glycosidase F. The calculated molecular weight of the cloned protein is 100kDa (secreted form) or 105kDa (full length protein). Based on amino acid composition, the estimated pI is 9.0 which is higher than the pI determined by 2-D gel

electrophoresis analysis (7.7-8.0) of purified ATX. This difference can be explained by the presence of sialic acid residues on the sugar moieties.

Autotaxin is secreted by A2058 human melanoma cells cultured in low abundance in serum-free conditioned medium. Autotaxin is a potent new cytokine with molecular mass 125 kDa which has been purified to homogeneity from the conditioned medium of the human melanoma cell line, A2058, utilizing sequential chromatographic methods as described herein. This new cytokine, termed autotaxin (ATX), is a basic glycoprotein with pI ~ 7.8. ATX is active in the high picomolar to low nanomolar range, stimulating both chemotactic and chemokinetic responses in the ATX-producing A2058 cells as well as other tumor cells. This motile response is abolished by pretreatment of the cells with pertussis toxin. ATX may therefore act through a G protein-linked cell surface receptor. These characteristics distinguish ATX from several small growth factors and interleukins which are implicated in tumor cell motility (Stracke et al., 1988; Ruff et al., 1985; Maciag et al., 1984; Gospodarowicz, 1984; Van Snick, 1990; Yoshimura 1987).

The protein of the present invention, which in one embodiment is derived from A2058 human melanoma cells, can be prepared substantially free from proteins with which it is normally associated using, for example, the purification protocol disclosed herein. Alternatively, the protein of the present invention can be prepared substantially free from proteins, by cloning and expressing the cDNA encoding autotaxin as disclosed herein.

A large volume of serum-free conditioned medium from appropriate producer cells (e.g., tumor cells) is collected and concentrated approximately 500-fold. This concentrated conditioned medium is then separated from other contaminating proteins by techniques that rely on

the chemical and physical characteristics of the protein. These include the molecular weight, relative hydrophobicity, net charge, isoelectric focusing point, and the presence of lectin-binding sugar residues on the protein.

Alternatively, the protein, or functional portion thereof, can be synthesized using chemical or recombinant means.

The protein of the present invention has a potent biological activity. Purified ATX is active in the picomolar range and 1 unit of activity corresponds to a concentration of approximately 500 pM as assessed by the cell motility assay described herein and elsewhere (Stracke et al., 1989).

The protein of the present invention has a molecular size, as determined by two dimensional gel electrophoresis, of from 120 to 130 kDa, or more specifically, about 125 kDa. Further, the protein of the present invention can have a pI in the range of 7.5 to 8.0, preferably, approximately 7.7. The present invention relates to autotaxin and peptides thereof having cell motility properties as described herein. These proteins and peptides thereof can be produced by isolation from a natural host or isolation as an expression product from a recombinant host.

The present invention also relates to a DNA segment coding for a polypeptide comprising an amino acid sequence corresponding to ATX, or a unique portion of such a sequence (unique portion being defined herein as at least 5, 10, 25, or 50 amino acids). In one embodiment, the DNA segment encodes any one of the amino acid sequences shown in SEQ ID NO:1 to SEQ ID NO:11 and SEQ ID NO:26 to SEQ ID NO:33. Another embodiment uses larger DNA fragments encoding amino acid sequences shown in SEQ ID NO:34, SEQ ID NO: 36 and SEQ ID NO:38. The entire coding region for autotaxin can also be used in the present invention shown

in SEQ ID NO:66 through SEQ ID NO:69.

In another embodiment, the present invention relates to a recombinant DNA molecule comprising a vector (for example plasmid or viral vector) and a DNA segment coding for a polypeptide corresponding to ATX, as can be prepared by one skilled in the art. Preferably, the coding segment is present in the vector operably linked to a promoter. The present invention also relates to a recombinant protein produced from a host cell expressing a cDNA containing a coding region of ATX. Examples of ATX cDNAs from a variety of sources have been cloned and can be used for expression, including *inter alia* A2058 carcinoma cells, N-tera 2D1 cells and human liver.

In a further embodiment, the present invention relates to a cell containing the above-described recombinant DNA molecule. Suitable host cells include procaryotic cells (such as bacteria, including E. coli) and both lower eucaryotic cells (for example, yeast) and higher eucaryotic cells (for example, mammalian cells). Introduction of the recombinant molecule into the host cells can be effected using methods known in the art.

In another embodiment, the present invention relates to a method of producing a polypeptide having an amino acid sequence corresponding to ATX. The method comprises culturing the above-described cells under conditions such that the DNA segment is expressed, and isolating ATX thereby produced.

In a further embodiment, the present invention relates to an antibody having affinity for ATX or peptide fragments thereof. The invention also relates to binding fragments of such antibodies. In one preferred embodiment, the antibodies are specific for ATX peptides having an amino acid sequence set forth in one of SEQ ID NO:1 through SEQ ID NO:11 and SEQ ID NO:26 through SEQ ID NO:34, SEQ ID NO: 36 and SEQ ID NO:38 through SEQ ID NO:52. In addition, the antibodies may recognize an

entire autotaxin protein.

Antibodies can be raised to autotaxin or its fragment peptides, either naturally-occurring or recombinantly produced, using methods known in the art.

The ATX protein and peptide fragments thereof described above can be joined to other materials, particularly polypeptides, as fused or covalently joined polypeptides to be used as carrier proteins. In particular, ATX fragments can be fused or covalently linked to a variety of carrier proteins, such as keyhole limpet hemocyanin, bovine serum albumin, tetanus toxoid, etc. See for example, Harper and Row, (1969); Landsteiner, (1962); and Williams et al., (1967), for descriptions of methods of preparing polyclonal antisera. A typical method involves hyperimmunization of an animal with an antigen. The blood of the animal is then collected shortly after the repeated immunizations and the gamma globulin is isolated.

In some instances, it is desirable to prepare monoclonal antibodies from various mammalian hosts. Description of techniques for preparing such monoclonal antibodies may be found in Stites et al., and references cited therein, and in particular in Kohler and Milstein (1975), which discusses one method of generating monoclonal antibodies.

In another embodiment, the present invention relates to an oligonucleotide probe synthesized according to the sense or antisense degenerative sequence set forth in one of SEQ ID NO:1 through SEQ ID NO:11, SEQ ID NO:26 through SEQ ID NO:33, SEQ ID NO:39 through SEQ ID NO:52, and SEQ ID NO:55 through SEQ ID NO:65.

Protein database searches of this sequence revealed a 45% amino acid identity with the plasma cell membrane marker protein, PC-1. ATX and PC-1 appear to share a number of domains, including two somatomedin B domains, the loop region of an EF hand, and the enzymatic site of

type I phosphodiesterase/ nucleotide pyrophosphatase. Like PC-1, ATX hydrolyzes p-nitrophenyl thymidine-5' - monophosphate, a type 1 phosphodiesterase substrate. This enzymatic function of ATX suggests a newly identified function for ecto/exo-enzymes in cellular motility.

In a further embodiment, the present invention relates to a method of diagnosing cancer metastasis and to a kit suitable for use in such a method. Preferably, antibodies to ATX can be used in, but not limited to, ELISA, RIA or immunoblots configurations to detect the presence of ATX in body fluids of patients (e.g. serum, urine, pleural effusions, etc.). These antibodies can also be used in immunostains of patient samples to detect the presence of ATX.

In yet another embodiment, the present invention relates to *in vivo* and *in vitro* diagnostics. ATX may be radiolabelled, by means known to one skilled in the art, and injected in cancer patients with appropriate ancillary substances also known to one skilled in the art, in order to ultimately detect distant metastatic sites by appropriate imagery. The level of ATX in tissue or body fluids can be used to predict disease outcomes and/or choice of therapy which may also include ATX inhibitors.

In a further embodiment, the present invention relates to a treatment of cancer. ATX antibodies can be cross-linked to toxins (e.g., Ricin A), by means known to one skilled in the art, wherein the cross-linked complex is administered to cancer patients with appropriate ancillary agents by means known to one skilled in the art, so that when the antibody complex binds to the cancer cell, the cell is killed by the cross-linked toxin.

In another embodiment, the different localizations of the normal versus tumorous forms of the ATX proteins within the tissue can be used as a tool for diagnosis and prognosis. The stage of disease progression can be monitored by elevated levels of ATX in the extracellular

space as opposed to its normal cell membranes association. In addition, treatment methods for control of tumor progression can be designed to specifically block the activity of the secreted form of ATX. Such methods would have a preferential effect upon secreted ATX during tumor progression while not effecting normal ATX formation.

Yet another embodiment utilizes the hot spot located in the region from approximately nucleotides 1670 through 1815, as a marker gene for identification of tissues carrying a tumorous form of ATX.

The present invention is described in further detail in the following non-limiting examples.

EXAMPLES

The following protocols and experimental details are referenced in the Examples that follow:

Materials. The polycarbonate Nuclepore membranes and the 48-well microchemotaxis chambers were obtained from Neuro Probe, Inc. Pertussis toxin (PT), ethylene glycol (biotechnology grade), methyl α-D-mannopyranoside were obtained from commercial vendors. The ampholyte, pH 3-10 Bio-Lyte and pH 8-10 Bio-Lyte, were obtained from Bio-Rad. Phenyl Sepharose CL-4B; affi-Gel concanavalin A; ZORBAX BioSeries-WAX (weak anion exchange) column (9.4mm x 24cm); Spherogel-TSK 4000SW, 3000SW and 2000SW columns (each 7.5mm x 30cm); the Pro-Pac PA1 (4 x 50mm) strong anion exchange column; the Aquapore RP300 C-8 reverse phase column (220 x 2.1mm); and the AminoQuant C-18 reverse phase column (200 x 2.1mm) were also obtained from commercial sources.

Affi-Gel 10 affinity resin was from Bio-Rad. The GeneAmp PCR Reagent kit with AmpliTaq and the GeneAmp RNA PCR kit were purchased from Perkin-Elmer. The 5' RACE kit came from Gibco BRL Life Technologies, Inc. The p-nitrophenyl thymidine-5'monophosphate was obtained from Calbiochem Biochemicals.

Ethylene glycol (biotechnology grade) was from Fisher

Biochemicals (Pittsburg, PA). Peptide N-glycosidase F ("PNGase F"), O-glycosidase, neuraminidase (*Arthrobacter ureafaciens*), and swainsonine ("Swn") came from Boehringer-Mannheim (Indianapolis, IN). 1-Deoxymannojirimycin ("dMAN"), and N-methyl-1-deoxynojirimycin ("NMdNM") were from Oxford GlycoSystems, Inc. (Rosedale, NY). Biotinylated concanavalin A, HRP-conjugated streptavidin, and HRP-conjugated goat anti-rabbit immunoglobulin were purchased from Pierce Chemicals (Rockford, IL). Polyvinyl pyrrolidone-free polycarbonate membranes and the microchemotaxis chamber were from NeuroProbe, Inc. (Cabin John, MD).

Cell Culture. The human melanoma cell line A2058, originally isolated by Todaro (Todaro et al., 1980), was maintained as previously described by Liotta (Liotta et al., 1986). The N-tera 2 (D1 clone) was a kind gift from Dr. Maxine Singer, Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health and was maintained as described (Andrews, P.W., Goodfellow, P.N. and Bronson, D.L. (1983) *Cell surface characteristics and other markers of differentiation of human teratocarcinoma cells in culture.*).

Production of Autotaxin. A2058 cells were grown up in T-150 flasks, trypsinized, and seeded into 24,000 cm^2 cell factories at a cell density of $1x10^{10}$ cells/factory. After 5-6 days, the serum-containing medium was removed and the cells were washed with DPBS. The factories were maintained in DMEM without phenol red, supplemented with 4 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml crystallized bovine serum albumin, 10 μg/ml bovine insulin, and 1 μM aprotinin. Culture supernatants were harvested every 3 days, frozen at -40°C and replaced with fresh serum-free medium. Each cycle of supernatant was tested for ATX production with a cell motility assay detailed below. Typically, a cell factory continued to be productive for 9-11 of these cycles.

After accumulation of approximately 45-60 L of supernatant, the culture supernatants were thawed and concentrated down to 2-2.5 L using an Amicon S10Y30 spiral membrane ultrafiltration cartridge. This supernatant was further concentrated in an Amicon high performance ultrafiltration cell using Diaflo membranes. The final volume achieved from 100-200 L of conditioned medium was typically 250-400 ml. All ultrafiltrations were performed at 4°C.

Cell Motility Assays. Purification of autotaxin was monitored by testing the motility-stimulating capacity of the fractions collected from the columns. These fractions were in buffers unsuitable for a chemotaxis assay so each fraction had to be washed into an appropriate buffer, i.e., 0.1% (w/v) BSA in DPBS containing calcium and magnesium. This dialysis was performed by adding aliquots of each fraction to be tested into Centricon-30™ ultrafiltration tubes, which retain molecular species larger than 30,000 daltons.

The assay to determine motility was performed in triplicate using a 48-well microchemotaxis chamber as described elsewhere in detail (Stracke et al., 1987; Stracke, et al., 1989). The Nuclepore™ membranes used in these modified Boyden chambers were fixed and stained with Diff-Quik.™ Chemotaxis was quantitated either by reading the stained membranes with a 2202 Ultroscan laser densitometer or by counting 5 randomly chosen high power fields (HPF) under light microscopy (400 x) for each replicate. Densitometer units (wavelength - 633 nm) have been shown to be linearly related to the number of cells per HPF (Taraboletti, 1987; Stracke, et al., 1989). Typically, unstimulated motility (background) corresponded to 5-10 cells/HPF and highly responding cells to 70-100 cells/HPF above unstimulated background (i.e., 75-110 total cells/HPF).

For experiments using PT, the toxin was pre-incubated

with the cells for 1-2 hr. at room temperature prior to the assay and maintained with the cells throughout the assay (Stracke, et al., 1987). The treated cells were tested for their motility response to the chemoattractant as well as for unstimulated random motility.

Purification of Autotaxin. Ammonium sulfate, to a final concentration of 1.2 M, was added to the concentrated A2058 conditioned medium for 1 hr. at 4°C. The solution was spun in a RC2-B Ultraspeed Sorvall centrifuge at 10,000 x g for 15 min. Only the supernatant had the capacity to stimulate motility.

In the first step, the sample was fractionated by hydrophobic interaction chromatography using 200 ml phenyl Sepharose CL-4B column equilibrated into 50 mM Tris (pH 7.5), 5% (v/v) methanol and 1.2 M ammonium sulfate. The supernatant from the ammonium sulfate fractionation was added to this column and eluted using linear gradients of 50 mM Tris (pH 7.5), 5% (v/v) methanol, with decreasing (1.2 - 0.0) M ammonium sulfate and increasing (0-50) % (v/v) ethylene glycol at 1 ml/min.

The active peak was pooled, dialyzed into 50 mM Tris, 0.1 M NaCl, 0.01 M $CaCl_2$, 20% (v/v) ethylene glycol, and subjected to a second fractionation by lectin affinity chromatography using a 40 ml Affi-Gel concanavalin A column run at 1 ml/min. The sample was eluted in a stepwise fashion in the same buffer with 0, 10, and 500 mM methyl α-mannopyranoside added successively. Fractions from each step of the gradient were pooled and tested for their capacity to stimulate motility.

In the third purification step, the sample that eluted at 500 mM α-methyl-mannopyranoside was dialyzed into 10 mM Tris (pH 7.5) with 30% (v/v) ethylene glycol and fractionated by weak anion exchange chromatography. Chromatography was carried out on a ZORBAX BioSeries-WAX column using a Shimadzu BioLiquid chromatograph and eluted with a linear gradient of (0.0 - 0.4 M) sodium chloride at

3 ml/min.

The active peak was pooled, dialyzed against 0.1 M sodium phosphate (pH 7.2), 10% (v/v) methanol, and 10% (v/v) ethylene glycol, and subjected to a fourth fractionation step on a series of Spherigel TSK columns (4000SW, 4000SW, 3000SW, 2000SW, in that order). This molecular sieve step was run using the Shimadzu BioLiquid chromatograph at 0.4 ml/min.

The active peak was pooled and dialyzed into 10 mM Tris (pH 7.5), 5% (v/v) methanol, 20% (v/v) ethylene glycol and subjected to a fifth (strong anion exchange) chromatography step, a Pro-Pac PA1 column run at 1 ml/min using a Dionex BioLC with AI450 software. The sample was eluted with a linear gradient of (0.0-0.4M) NaCl.

In order to calculate activity yields after each step of purification, a unit of activity had to be derived. The dilution curve of ATX was biphasic with a broad peak and a linear range at sub-optimal concentrations. One unit of activity/well (i.e., 40 units/ml) was defined as 50% of the maximal activity in a full dilution curve. This allowed calculation of the activity contained in any volume from the dilution needed to achieve 1 unit/well. Therefore, if a 1:10 dilution were needed in order to produce 1 unit of activity/well, the material contained 10 x 40 = 400 units/ml.

Gel Electrophoresis. Protein samples were analyzed by SDS-polyacrylamide gel electrophoresis using the conditions of Laemmli (Laemmli, 1970). In brief, 7 or 8% SDS-containing polyacrylamide gels were prepared or pre-poured (8-16%) gradient gels were obtained commercially. Samples were prepared with or without reducing conditions (5% β-mercaptoethanol). After electrophoretic separation, the gels were stained using Coomassie Blue G-250 as previously described (Neuhoff, et al., 1988). In this staining protocol, which ordinarily requires no destaining step, the Coomassie stain appears to be able to stain as little

as 10 ng of protein.

For two-dimensional electrophoresis, the protein, in 20% ethylene glycol, was dried in a Speed-vac and redissolved in loading solution: 9M urea, 1% (v/v) pH 3-10 Bio-Lyte, and 2.5% (v/v) Nonidet-P40. This sample was then subjected to isoelectric focusing (O'Farrell, 1975) using a Bio-Rad tube cell in 120 x 3 mm polyacrylamide tube gels containing 9M urea, 2% (v/v) pH 3-10 Bio-Lyte, 0.25% (v/v) pH 8-10 Bio-Lyte and 2.5% (v/v) Nonidet-P40. Reservoir solutions were 0.01 M phosphoric acid and 0.02 M NaOH. Non-equilibrium isoelectric focusing (O'Farrell, et al., 1977) was run initially with constant voltage (500 v) for 5 hr. Since the protein was basic, the procedure was repeated under equilibrium conditions (500 v for 17 hr.). Electrophoresis in the second dimension was performed on a 7.5% polyacrylamide using the conditions of Laemmli (1970). The gel was stained with Coomassie Blue G-250 as above.

Preparation of peptides for internal sequence of autotaxin. Homogeneous ATX was sequentially digested with cyanogen bromide and, following reduction and pyridylethylation, with trypsin (Stone, et al., 1989). The resulting fragments were then separated by gradient elution on an Aquapore RP300 C-8 reverse phase column: 0.1% (v/v) trifluoroacetic acid and (0-70)% acetonitrile over 85 min. at a flow rate of 0.2 ml/min. A Dionex AI450 BioLC system was utilized and fractions were collected manually while monitoring the absorbance at 215 nm.

Sequence analysis of peptides. The amino acid sequences of peptides resulting from digestion and purification of ATX peptides #1-7 and 12-18, corresponding to SEQ ID NO:1 through SEQ ID NO:7 and SEQ ID NO:26 through SEQ ID NO:32, respectively, were determined on a Porton Instruments 2020 off-line sequenator using standard program #1. Phenylthiohydantoin amino acid analysis of sequenator runs were performed on a Beckman System Gold HPLC using a

modified sodium acetate gradient program and a Hewlett-Packard C-18 column. ATX-100 (SEQ ID NO:8), ATX-101 (SEQ ID NO:9), ATX-102 (SEQ ID NO:10), ATX-103 (SEQ ID NO:11) and ATX 104 (SEQ ID NO:33) were sequenced from gel-purified ATX.

Protein databases (Pearson, et al. 1988) that were searched for homologies in amino acid sequence with the ATX peptides include: GenBank (68.0), EMBL (27.0), SWISS-PROT (18.0), and GenPept (64.3).

EXAMPLE 1

Purification of Autotaxin

The A2058 cells had been previously shown to produce protein factors which stimulate motility in an autocrine fashion (Liotta, et al., 1986). Conditioned medium from these cells was therefore used to identify and purify a new motility-stimulating factor, which is here named autotaxin and referred to as ATX. Since the purification was monitored with a biological assay, motility-stimulating activity had to be maintained throughout. The activity proved to be labile to freezing, acidic buffers, proteases (but not DNase or RNase), reduction, strong chaotrophic agents (e.g. > 4 M urea), and a variety of organic solvents (isopropanol, ethanol, acetonitrile). An organic solvent, ethylene glycol, which did not decrease bioactivity, was added for both storage and chromatographic separation.

100-200 L of serum-free conditioned medium were required in order to produce enough ATX for amino acid sequence analysis. The medium contained low concentrations of both BSA (5 μg/ml) which was needed as a carrier protein and insulin (10 μg/ml) which was required to support cell growth in low protein medium. Ultrafiltration to concentrate this large volume was performed with low protein-binding YM30 membranes which retain molecular species with M_r > 30,000. As seen in Table 1, 200 L of conditioned medium prepared in this

manner resulted in 10 x 10^6 units of activity. However, the initial unfractionated conditioned medium contained additional substances known to stimulate activity, particularly insulin, which does not completely wash out in the ultrafiltration step and which is additive to the motility stimulating activity in a complex manner (Stracke, et al., 1989). This had to be taken into account in order to determine yields for subsequent steps in which insulin had been removed.

TABLE 1. PURIFICATION OF AUTOTAXIN

Purification Step	Protein (mg)	Activity [a] (total units)	Specific Activity (units/mg)	Recovery (%)[b]
200 L Conditioned Medium	33,000	10,000,000[c]	300	
Phenyl Sepharose	1,235	460,000	370	100
Concanavalin A	58	660,000	11,400	100
Weak Anion Exchange	4.5	490,000	110,000	100
TSK Molecular Sieves	~0.4[d]	220,000	550,000	48
Strong Anion Exchange	~0.04[d]	24,000[c]	600,000	5.2

[a] Activity calculated from Boyden chamber assay. The dilution which resulted in 50% of maximal activity (generally approximately 20 laser density units or ~40 cells/HPF) was chosen to have 1 unit of activity per well (equivalent to 40 units/ml).

[b] Recovery was estimated from activity, after the first purification column (i.e., phenyl sepharose).

[c] Initial activity in the unfractionated conditioned medium reflected the fact that insulin was used in the medium as a necessary growth factor under low protein conditions.

[d] Estimated protein is based on quantification by amino acid analysis.

[e] This specific activity for purified protein corresponds to ~ 10 fmol ATX/unit of motility activity (in a Boyden chamber well).

The first step in the purification involved fractionation by hydrophobic interaction chromatography using a phenyl Sepharose CL-4B column. The results are shown in Figure 1. Most proteins, including insulin, eluted from the column in early fractions or in the void. However, the peak of activity eluted relatively late. The activity which was purified was estimated as 460,000 units ± 20% (Table 1). As the pooled peak of activity from the phenyl Sepharose fractionation is considered to be the first sample without significant insulin contamination, subsequent yields are measured against its total activity. Gel electrophoresis of a small portion of the pooled peak of activity (Figure 6A, column 2) revealed a large number of protein bands with BSA predominant from the original conditioned medium.

In the second step of purification, the active peak was applied to the lectin affinity column, Affi-Gel concanavalin A. As shown in Figure 2, most protein (estimated to be 90% of the total absorbance at 280 nm) failed to bind to the column at all. The non-binding fraction contained essentially no motility-stimulating activity (see dotted line in Figure 2). When a linear gradient of methyl α-D-mannopyranoside was applied to the column, chemotactic activity eluted off in a prolonged zone, beginning at a concentration of approximately 20 mM sugar. Consequently, a step gradient was used to elute. Pure BSA failed to bind to con A.

Activity was found primarily in the 500 mM step of methyl α-D-mannopyranoside. There appeared to be no significant loss of activity as seen in Table 1; however, specific activity (activity/mg total protein) increased thirty-fold. Gel electrophoresis of the pooled and

concentrated peak (Figure 6A, column 3) revealed that the BSA overload was no longer apparent and the number of bands were much reduced. When the unbound protein was concentrated and applied to a gel, it appeared identical to the active peak from phenyl Sepharose-4B with a large BSA band.

The third purification step involved fractionating the previous active peak by weak anion exchange chromatography as shown in Figure 3. Under the running conditions, activity eluted in a broad peak-shoulder or double peak in the middle of the shallow portion (0.0-0.4 M) of the NaCl gradient. The largest proportion of protein, lacking in motility-stimulating capacity, bound strongly to the column and eluted off in high salt (1 M NaCl). There appeared to be no significant loss of activity, though specific activity increased by twenty-fold (Table 1). Analysis by gel electrophoresis of both the peak (28-34 min. in Figure 3) and the shoulder (35-45 min. in Figure 3) is shown in Figure 6A (columns 4 and 5, respectively). Two predominant protein bands resulted: a broad doublet around 25-35 kDa and a second doublet around 110-130 kDa.

In the fourth purification step, the active peak was applied to a series of molecular sieves. Spectrophotometric monitoring of the eluant revealed two large peaks of protein (Figure 4). Activity corresponded to the first, higher molecular weight peak. Recovery of activity was ~48% with a five-fold increase in specific activity. Analysis by gel electrophoresis was performed under non-reducing and reducing conditions as shown in Figure 6B (columns 2 and 3, respectively). This fractionation step had essentially removed all contaminating protein of molecular weight < 55 kDa. The predominant band remaining has a molecular weight of 120 kDa unreduced and 125 kDa reduced; there are two minor bands with molecular weights 85 kDa and 60 kDa. The fact

that the 120 kDa protein changes so little in electrophoretic mobility after reduction would tend to indicate a paucity of disulfide bonds. However, the existing disulfide bonds have functional significance because motility-stimulating activity is labile to reduction.

The fifth purification step involved fractionation of the active peak by strong anion exchange chromatography. As shown in Figure 5, activity corresponds to two broad optical absorbance peaks in the middle of the gradient with contaminating proteins eluting earlier. These two peaks were identical by amino acid analysis and by polyacrylamide gel electrophoretic separation. They presumably represent different glycosylation states of the same parent protein. Activity is shown in Figure 5 at two different sample dilutions. Several dilutions of the fractionated samples were often necessary in order to resolve the true "peak" of activity as the shape of the ATX dilution curve was not sharp due to saturation and down regulation at high concentrations. Recovery from this chromatographic step is lower (5% compared to phenyl Sepharose), as might be expected when a minute quantity of protein is applied to a column; however, specific activity again increased (Table 1). Analysis by gel electrophoresis revealed a single protein band at molecular weight 120 kDa, unreduced (Figure 6C, column 2).

EXAMPLE 2

Characterization of Autotaxin

Two dimensional gel electrophoresis of the purified protein (Figure 7) revealed a single predominant band. The band slopes downward slightly toward the basic side of the gel in a manner that is characteristic of glycosylated proteins. A basic pI of 7.7 $\pm$ 0.2 was essentially the same whether the isoelectric focusing was run under non-equilibrium conditions (5 hr.) or was allowed to go to equilibrium (17 hr.).

A dilution curve of the purified protein is shown in Figure 8. The protein is active in the picomolar range and 1 unit of activity appears to correspond to a concentration of 400-600 picomolar (or approximately 10 fmol of ATX/Boyden chamber well). When dilutions were begun at higher concentrations of ATX, the resultant curve showed a broad plateau with down-regulation at the highest concentrations. The motility response to purified autotaxin is highly sensitive to pertussis toxin (hereinafter referred to as "PT") (Table 2 and Figure 9) with approximately 95% inhibition of activity at 0.5 μg/ml PT.

TABLE 2. Effect of Pertussis Toxin (PT) on Autotaxin-stimulated motility

	A2058 Motility Response (density units[1])	
	control cells[2]	Pertussis toxin-treated cells[3]
Condition medium[4]	60.3	0.4
Purified Autotaxin	38.5	0.0

[1] Chemotaxis quantitated by motility assay (Stracke, et al., 1978).

[2] A2058 cell suspended at 2 x 10^6 cells/ml in DMEM supplemented with 1 mg/ml bovine serum and rocked at room temperature for 1 hr.

[3] As control with 0.5 μg/ml pertussis toxin.

[4] Prepared by adding DMEM without phenol red supplemented with 0.1 mg/ml bovine serum albumin to subconfluent flasks of A2058 cells. The medium was harvested after 2 days incubation at 37°C in a humidified atmosphere and concentrated 25-30 fold using an Amicon ultrafiltration assembly with a YM-30 membrane.

Checkerboard analysis was performed to assess the random (chemokinetic) versus the directed

(chemotactic) nature of the motility response to ATX. Chambers were assembled with different concentrations of ATX above and below the filter, using ATX purified through the weak anion exchange fractionation step. Squares below the diagonal reflect response to a positive gradient, squares above reflect response to a negative gradient, and squares on the diagonal reflect random motility in the absence of a gradient. ATX stimulates both chemotactic and chemokinetic responses (Figure 10), with chemotactic responses as high as fifteen-fold above background and chemokinesis as high as eight-fold above background.

Amino acid analysis after complete acid hydrolysis was used to quantitate purified protein. This hydrolysis was carried out on protein excised from a polyacrylamide gel and presumed to be pure. The analysis indicated that 2.7 nmol of protein was present after fractionation on the molecular sieve. After fractionation by strong anion exchange chromatography, approximately 300 pmol remained. The results of the analysis are shown in Table 3.

TABLE 3. AMINO ACID COMPOSITION OF AUTOTAXIN
(CYS and TRP were not determined in this analysis)

Amino Acid	Residues/100
ASX	12.5
THR	6.0
SER	5.7
GLX	9.4
PRO	7.4
GLY	7.0
ALA	3.9
VAL	6.7
MET	1.2
ILE	4.3
LEU	9.0
TYR	5.2
PHE	5.2
HIS	3.8
LYS	7.4
ARG	5.4

T,310

EXAMPLE 3

ATX Degradation and Determination of Amino Acid Sequence

Attempts to obtain N-terminal sequence information from purified ATX repeatedly proved futile. The purified protein was therefore, sequentially digested and the resulting peptides fractionated by reverse phase chromatography. The results are shown in Figure 11. Multiple sharp peaks including clusters at both the hydrophilic and hydrophobic ends of the gradient are seen.

Several of these peptide peaks were chosen randomly for Edman degradation and N-terminal amino acid sequence analysis. Seven of the eight peaks (shown in Figure 11) chosen gave clear single sequence information as seen in Table 4. Using material from a separate digestion and purification, the remaining four sequences were also obtained.

Separate sense and antisense oligonucleotide probes were synthesized according to the fragment sequences of Table 4 by methods known to one skilled in the art. Representative probes are shown in Table 5.

TABLE 4. Peptide sequences for Autotaxin.

PEPTIDE NO.	AMINO ACID SEQUENCE	SEQ ID: NO:	NAME
1.	WHVA	SEQ ID NO:1	ATX 18
2.	PLDVYK	SEQ ID NO:2	ATX 19
3.	YPAFK	SEQ ID NO:3	ATX 20
4.	QAEVS	SEQ ID NO:4	ATX 24
5.	PEEVTRPNYL	SEQ ID NO:5	ATX 29
6.	YDVPWNETI	SEQ ID NO:6	ATX 47
7.	VPPFENIELY	SEQ ID NO:7	ATX 48
8.	GGQPLWITATK	SEQ ID NO:8	ATX 100

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9.	VNSMQTVFVGY-GPTFK	SEQ ID NO:9	ATX 101
10.	DIEHLTSLDFFR	SEQ ID NO:10	ATX 102
11.	TEFLSNYLTNVDD-ITLVPETLGR	SEQ ID NO:11	ATX 103
12.	QYLHQYGSS	SEQ ID NO:26	ATX 37
13.	VLNYF	SEQ ID NO:27	ATX 39
14.	YLNAT	SEQ ID NO:28	ATX 40
15.	HLLYGRPAVLY	SEQ ID NO:29	ATX 41
16.	SYPEILTPADN	SEQ ID NO:30	ATX 44
17.	XYGFLFPPYLSSSP	SEQ ID NO:31	ATX 53
18.	TFPNLYTFATGLY	SEQ ID NO:32	ATX 59
19.	VNVISGPIFDYDYDGLH DTEDK	SEQ ID NO:33	ATX 104

Peptide numbers 1-7 refer to peaks numbered in Figure 11. Peptide numbers 12-18 refer to peptides purified from the preparation which yielded peptide numbers 1-7. Peptides 8-11 and 19, are from a separate purification, not shown in Figure 11.

X refers to potentially glycosylated residues.

TABLE 5.

Oligonucleotides synthesized from peptide sequences of autotaxin (ATX). The number of the oligonucleotide corresponds to the ATX peptide number as per Table 4. The final letter suffix distinguishes whether the oligonucleotide is a sense (S) or antisense (A) sequence.

Oligo	Sequence	SEQ ID NO:
A-18A	GTT-GGC-AGC-NAC-RTG-CCA	SEQ ID NO:12
A-18S	TGG-CAY-GTN-GCT-GCC-AAC	SEQ ID NO:13
A-20A	CTT-GAA-GGC-AGG-GTA	SEQ ID NO:14
A-20S	TAY-CCT-GCN-TTY-AAG	SEQ ID NO:15
A-29A	GGT-NAC-YTC-YTC-AGG	SEQ ID NO:16

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A-29S	CCT-GAR-GAR-GTN-ACC	SEQ ID NO:17
A-47A	NGT-NGC-RTC-RAA-TGG-CAC-RTC	SEQ ID NO:18
A-47S	GAY-GTG-CCA-TTY-GAY-GCN-ACN	SEQ ID NO:19
A-48A	GTT-DAT-RTT-STC-RAA-TGG-GGG	SEQ ID NO:20
A-48S	CCC-CCA-TTT-GAG-AAC-ATC-AAC	SEQ ID NO:21
A-100A	CTT-NGT-NGC-NGT-DAT-CCA-NAR-GGG-YTG-GCC-GCC	SEQ ID NO:22
A-100S	GGC-GGC-CAR-CCC-YTN-TGG-ATH-ACN-GCN-ACN-AAG	SEQ ID NO:23
A-101A	CTT-RAA-GGT-GGG-GCC-RTA-GCC-CAC-RAA-GAC-TGT-YTG-CAT	SEQ ID NO:24
A-101S	ATG-CAR-ACA-GTC-TTY-GTG-GGC-TAY-GGC-CCC-ACC-TTY-AAR	SEQ ID NO:25

EXAMPLE 4

Antipeptide Antibodies

Rabbits were injected with ATX-101 (SEQ ID NO:10) which had been cross-linked to bovine serum albumin. Antisera from these rabbits was subjected to salt precipitation followed by purification using affinity chromatography with Affi-Gel 10 beads covalently linked to the peptide, ATX-101 (SEQ ID NO:10). This affinity purified antibody reacted with the partially purified protein on immunoblots. This same antibody has been used to perform immunohistochemical stains on human tissue.

EXAMPLE 5

Enzymatic Deglycosylation of ATX

Purified ATX that was to be treated with peptide N-glycosidase F (PNGase F) was first dialyzed into 0.2 M sodium phosphate, 10% (v/v) ethylene glycol pH 7.0, using Centricon-30 ultrafiltration tubes. Varying concentrations of PNGase F were added to the ATX and incubated 16-18 hr. at 37°C. Complete digestion appeared

to occur at concentrations of enzyme above 30 mU/ml (where 1 U converts 1mmol of substrate/min). For comparison, the experiments were repeated in the presence of 0.1 M β-mercaptoethanol or 0.1% (w/v) SDS plus 0.5% (v.v) Nonidet-P40. ATX that was to be treated with neuraminidase or O-glycosidase was dialyzed into 20 mM sodium phosphate, 0.1 M calcium acetate, and 10% (v/v) ethylene glycol (pH 7.2). Neuraminidase was added to a final concentration of 2 U/ml. For treatment with neuraminidase alone, this mixture was incubated 16-18 hr at 37°C. Since O-glycosidase requires the removal of terminal sialic acid residues for efficient deglycosylation, ATX was pre-incubated with neuraminidase for 30-125 mU/ml and incubated 16-18 hr. at 37°C. The treated ATX was then dialyzed into 50 mM Tris with 20% ethylene glycol for storage at 5%C.

Treatment of ATX with N-glycosylation altering agents

A2058 cells were split into four 150 cm^2 flasks and incubated until just subconfluent in DMEM supplemented with 10% fetal calf serum. The medium was then replaced with fresh 10% FCS/DMEM to which had been added DPBS for control, 1mM dMAN, 1 mM NMdNM, or 10 mM (1.7 mg/ml) Swn. Concentrations of these pharmacological agents were similar to those previously described as inhibiting N-glycan processing enzymes in melanoma cells (Seftor, et al. 1991; Dennis, et al. 1990) as well as carcinoma cells (Ogier, et al. 1990). On the next day, each flask was washed twice with Dulbecco's phosphate buffered saline with calcium ("DPBS") then 20 ml of Dulbecco's minimum essential medium ("DMEM") supplemented with 0.01% (w/v) bovine serum albumin ("BSA") was added. The same concentration of each agent was added to the appropriate equilibrated flask and incubated for ~ 24 hr, after which the medium from each treatment group was collected, concentrated, washed into DPBS and stored at 5°C.

Cells from each flask were trypsinized and

counted. There was no loss of viability or reduced cell number in any of the treatment groups compared to control cells.

Effect of PNGase F on ATX

ATX binds to concanavalin A ("Con A") agarose beads and is eluted with buffer containing 0.5 M methyl a-D-mannopyranoside, indicating that ATX is likely to contain mannose residues. Such mannose sugar residues are most characteristic of N-linked oligosaccharides. In order to verify that ATX contained asparagine-linked oligosaccharides, we treated it with the endoglycosidase, PNGase F, which cleaves high mannose, hybrid, and complex N-linked oligosaccharides at the asparagine residue.

Partially purified ATX was treated with 60 mU/ml of enzyme under a variety of increasingly denaturing conditions and then separated by polyacrylamide gel electrophoresis (Figure 16). Lane 1 shows untreated material; the 125 kDA band (arrow) is autotaxin. When this material is treated overnight with PNGase F under very mild conditions, the size of the 125 kDa band decreases to ~100- 105 kDa. Addition of 0.1 M b-mercaptoethanol (Lane 2) or 0.5% Nonidet-P40 (lane 3) to the ATX sample has no effect on the size of the resultant protein band. Even complete denaturation of ATX of boiling the sample for 3 min in 0.1% SDS with (lane 5) or without (lane 4) β-mercaptoethanol, followed by addition of 0.5% Nonidet-P40 to maintain enzymatic activity, has no effect on the final size of deglycosylated protein, indicating that the deglycosidation reaction was complete even under mild conditions.

Because these results showed that ATX contained N-linked oligosaccharide groups, it became important to see if these sugar moieties were necessary for stimulation of motility. The partially purified ATX sample was treated with varying concentrations of PNGase F (0.1 to 60 mU/ml) under mild, non-denaturing conditions. Analysis of

the resulting digest by polyacrylamide gel electrophoresis is shown in Figure 17A. As this figure shows, the digestion was incomplete using from 0.1 to 10 mU/ml of enzyme and resulted in a smear of protein between 100-125 kDa. However, at higher concentrations of enzyme, cleavage of N-linked oligosaccharides from ATX appears to be complete. When these different digestion products were compared for their capacity to stimulate motility (Figure 17B), there was no significant difference between groups.

EXAMPLE 6

Cloning the 3' end of Autotaxin (4C11)

ATX is active in picomolar to nanomolar concentrations and is synthesized in very small concentrations by A2058 cells. As might be expected, the cDNA clone was relatively rare, requiring various strategies and multiple library screenings in order to identify it (Figure 12). Attempts to utilize degenerate oligonucleotides deduced from known peptide sequences were unsuccessful--whether we used the oligo nucleotides for screening cDNA libraries or for reverse transcription of mRNA followed by amplification with the polymerase chain reaction (RT/PCR). We then utilized an affinity-purified anti-peptide ATX-102 antibodies to screen an A2058 expression library.

These anti-peptide antibodies were generated by methods well established in the art and described previously with slight modification (Wacher, et al., 1990). In brief, the previously identified peptide, ATX-102 (Stracke, et al., 1992), was synthesized on a Biosearch 9600 peptide synthesizer. It was then solubilized in 1X PBS containing 20% (v/v) DMSO and conjugated to the protein carrier, bovine serum albumin (BSA), with glutaraldehyde. For the first injection into New Zealand white rabbits, the BSA-peptide conjugate was emulsified with complete Freund's adjuvant and injected subcutaneously. For subsequent injections, the BSA-

peptide conjugate was emulsified with incomplete Freund's adjuvant. The resultant antiserum was heat-inactivated at 56°C for 30 min. Immunoglobulins were precipitated out in 47% saturated ammonium sulfate, then redissolved and dialyzed into PBS. Antibodies were adsorbed onto peptide-conjugated Affi-Gel 10 resin (made using the BioRad protocol), eluted with 0.1 N acetic acid, and neutralized with 2 M Tris-HCl, pH 8. The resulting affinity-purified antibodies were dialyzed into DPBS, concentrated, aliquotted, and stored at -20°C. The antibodies were found to recognize a 125 kDa protein on immunoblots of partially purified A2058 conditioned medium and to preferentially stain some breast carcinoma cells compared to normal breast using immunohistochemical techniques.

An A2058 cDNA library was prepared by purifying poly-A purified mRNA from the cells then size-selecting mRNA > 1000 bp for the preparation of cDNA. The cDNA inserts were placed into λgt11 directionally, using the ProMega cDNA kit using standard methods well-established in the field. LE 392 cells were infected with the λgt11 and plaques were transferred onto nitrocellulose membranes by overnight incubation at 37°C. The antibody was incubated with the membranes in blocking buffer for 2 hr at room temperature, using approximately twice the concentration of antibody which gave a strong response on Western blot analysis. Secondary antibody was goat anti-rabbit immunoglobulin, and the blot was developed colorimetrically with 4-chloro-1-naphthol.

Positive clones were confirmed by antibody competition with specific peptides but not unrelated peptides. Using this technique and multiple subclonings, we obtained a partial cDNA clone of the autotaxin gene, which we called 4C11. The 4C11 insert was removed from λgt11 by restriction enzyme digests and subcloned into pBluescript for sequencing by standard Sanger techniques (Sanger, et al., 1977). The 4C11 clone contained bases,

including the poly-adenylated tail and the AATAAA adenylation signal locus, i.e., it contained the 3' terminus of the gene. It also included a 627 base open reading frame. Database analysis of this nucleotide sequence revealed that it is unique. The predicated amino acid sequence for 4C11 is 209 amino acids long with exact matches for 7 previously identified ATX peptides: (ATX-20, ATX-34, ATX-102, ATX-104, ATX-204, ATX-215, and ATX-244).

EXAMPLE 7

Cloning the 5' terminus of ATX

Database analysis of the 3' terminus of the ATX gene demonstrated a novel protein. However, we have found an interesting homology that has helped to guide us in exploring its function. ATX had a 45% amino acid identity and a 57% nucleotide identity with PC-1, a marker of B cell activation found on the surface of plasma cells. Using the PC-1 protein sequence as a guide, we found that ATX peptide homologies were scattered throughout the length of the protein. The only exception was the far amino terminus of PC-1, which includes the transmembrane and intracellular domains, and which had no homologies. Knowing approximate localization of the ATX peptides along the length of ATX, we then amplified different segments of ATX by the PCR (Figure 13). These amplified segments of DNA were then subcloned into plasmids utilizing the TA Cloning kit of ProMega. The PCR amplified DNA could then be sequenced using standard Sanger sequencing techniques (Sanger, et al., 1977).

Cloning of full length ATX gene

A reverse transcriptase reaction was performed using total or oligo-(dT) purified RNA from A2058 or N-tera 2D1 cells as template and an anti-sense primer from the 5' end of 4C11 (GCTCAGATAAGGAGGAAAGAG) (SEQ ID NO:55). This was followed by one or two PCR amplification of the resultant cDNA using the commercially available kit from Perkin-Elmer and following manufacturer's directions.

These PCR reactions utilized nested antisense primers from 4C11 (GAATCCGTAGGACATCTGCTT (SEQ ID NO:56) and TGTAGGCCAAACAGTTCTGAC (SEQ ID NO:57)) as well as degenerate, nested sense primers deduced from ATX peptides: ATX-101 (AAYTCIATGCARACIGTITTYGTIG (SEQ ID NO:58) and TTYGTIGGITAYGGICCIACITTYAA (SEQ ID NO:59)), ATX-103 (AAYTAYCTIACIAAYGTIGAYGAYAT (SEQ ID NO:60) and GAYGAYATIACICTIGTICCIGGIAC (SEQ ID NO:61)), or ATX-224 (TGYTTYGARYTICARGARGCIGGICCICC (SEQ ID NO:62)). The amplified DNA was then purified from a polyacrylamide gel using standard procedures and ligated into the pCR™ plasmid using the TA cloning kit (Invitrogen Corporation) according to manufacturer's directions.

The 5' RACE kit was utilized to extend the 5' end of ATX cDNA using total RNA from N-tera 2D1 as template and previously obtained sequence as primer (GCTGTCTTCAAACACAGC) (SEQ ID NO:63). The 5' end of the A2058 synthesized protein was obtained by using previously obtained sequence as primer (CTGGTGGCTGTAATCCATAGC) (SEQ ID NO:64) in a reverse transcriptase reaction with total A2058 RNA as template, followed by PCR amplification utilizing the 5' end of N-tera 2D1 sequence as sense primer (CGTGAAGGCAAAGAGAACACG) (SEQ ID NO:65) and a nested antisense primer (GCTGTCTTCAAACACAGC) (SEQ ID NO:63). A2058 DNA encoding ATX is set forth in a SEQ ID NO:68 and the amino acid sequence is provided in SEQ ID NO:69.

DNA sequencing: DNA sequencing was performed using dideoxy methodology (Sanger, et al. 1977) and (^{35}S)dATP (Du Pont, New England Nuclear).

We have found one region between the 5' end of the 4C11 and the ATX peptide designated ATX-101, also referred to as the "hot spot". This region has been sequenced five times with different sequences found each time. The hot spot appears to be located within the region from approximately nucleotide 1670 to 1815. The consensus sequence is represented by amino acids position 559 through 604. Variations found include DNA sequence

that results in single and multiple amino acid insertions. One sequence had a stop codon in this region and may have represented an intron. This region has been found to be variable in forms of ATX.

EXAMPLE 8

Cloning ATX in a human teratocarcinoma cell line

The fact that ATX is present in other cancer cells was confirmed by sequence information from N-tera 2D1, a human teratocarcinoma cell line. For these cells, a prepared cDNA library in λgt10 was amplified and the cDNA inserts were extracted. Using oligonucleotide primers based on known A2058 ATX sequence, DNA segments were amplified by PCR. The DNA segments were then subcloned into plasmids and sequenced as for A2058. We have 3104 bp DNA sequence for N-tera ATX (SEQ ID NO:66) and smaller portions thereof. This includes an open reading frame that codes for a putative protein containing 861 amino acids (SEQ ID NO:67) and smaller portions thereof. Like the A2058 ATX, the N-tera 2D1 sequence has homologies for multiple ATX peptides (Figure 15). Sequence homology between the A2058 and N-tera 2D1 cells is approximately 99%.

EXAMPLE 9

Cloning 5′ end of ATX in human normal liver

The 5′ end of ATX has proven difficult to obtain from either tumor cell line to date. Normal human liver mRNA was therefore amplified using the 5′ RACE kit (Clontech) with known sequence from A2058 ATX as antisense primer. A DNA segment was obtained and has been sequenced. This segment codes for 979 amino acids, including an initiating methionine (SEQ ID NO:38). The putative protein sequence also includes a 20 amino acid transmembrane domain which is different from the tumor ATX's (SEQ ID NO:54), as shown in Table 7. Both tumorous forms of ATX apparently lack a transmembrane region and are instead secreted proteins.

Table 7

Nucleotide and Amino Acid Sequences Encoding Liver ATX Amino Terminus containing the Transmembrane region

Protein Sequence (SEQ ID NO: 54)

Met Ala Arg Arg Ser Ser Phe Gln Ser Cys Gln Asp Ile Ser Leu Phe Thr Phe Ala Val Gly Val Asn Ile Cys Leu Gly Phe Thr Ala His Arg Ile Lys Arg Ala Glu Gly Trp

T,420

DNA Sequence (SEQ ID NO: 53)

ATGGCAAGGA GGAGCTCGTT CCAGTCGTGT CAAGATATAT CCCTGTTCAC
TTTTGCCGTT GGAGTCAATA TCTGCTTAGG ATTCACTGCA CATCGAATTA
AGAGAGCAGA AGGATGG

EXAMPLE 10

Domains of ATX

Searches of protein databases (Pearson, et. al. 1988) confirmed that the homology between ATX and PC-1 was present throughout the length of the extracellular portion of the molecules (Buckley, et. al., 1990; Funakoshi, et. al. 1992). There is a 45% amino acid identity and a 64% similarity between the 2 protein sequences (Fig. 18). For the cDNA sequence, the identity is ~57%.

These proteins share several interesting properties and domains (Fig. 19). Both have a number of potential N-linked glycosylation sites: four for ATX (Asn54, Asn463, Asn577, Asn859) and nine for PC-1. Both have adjacent somatomedin B domains near the amino end of the extracellular domain. This somatomedin B domain is a cysteine-rich region containing 3 presumed cystine cross-

linkages. ATX has 33 Cys residues and PC-1 has 37; 30 of these Cys residues are identical in placement. Both proteins also contain the loop region of an EF hand (Buckley, et. al. 1990; Kretsinger, 1987). In addition, both proteins have a transmembrane/signal peptide region with a short intracellular peptide, common in ectoenzymes (Maroux, 1987). However, the amino acid identity between ATX and PC-1 in the intracellular and transmembrane regions is only 11%.

Finally, both proteins have a region homologous to the bovine intestinal phosphodiesterase enzymatic domain with conversation of the threonine that is thought to act as the intermediate phosphate binding site (Culp, et al. 1985). PC-1 has been demonstrated to have phosphodiesterase type I, nucleotide pyrophosphatase, and threonine-specific kinase enzymatic activities (Rebbe, et al. 1991; Oda, et al. 1991). In order to test whether purified ATX had type I phosphodiesterase activity, samples were incubated with p-nitrophenyl thymidine-5'-monophosphate at pH 8.9 for 30 min. Samples were assayed in a 100 μl volume containing 50 mM Tris-HCl, pH 8.9 and 5 mM p-nitrophenyl thymidine-5'-monophosphate. After a 30 minute incubation at 37 °C the reactions were terminated by addition of 900 ml 0.1 N NaOH and the amount of product formed was determined by reading the absorbance at 410 nm. ATX was found to hydrolyze the p-nitrophenyl thymidine-5'-monophosphate (Razzell, 1963) at a rate of 10 pmol/ng/min, a reaction rate similar to that reported for PC-1 (Oda, et al. 1993).

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All publications mentioned hereinabove are hereby incorporated in their entirety by reference.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the present invention and appended claims.